INVITRO AND INVIVO EVALUATION OF *ALLIUM SATIVUM* AND *ZINGIBER OFFICINALIS* EXTRACTS MEDIATED GLYCEROSOMES FOR ANTIMICROBIAL AND ANTI-ARTHRITIC POTENTIAL

SYNOPSIS

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Introduction

Garlic has been used for centuries in various societies to combat infectious disease. Garlic (*Allium sativum*) is a species in the onion genus, *Allium*. Its close relatives include the onion, shallot, leek, chive, and Chinese onion. Garlic is native to Central Asia and northeastern Iran, and has long been a common seasoning worldwide, with a history of several thousand years of human consumption and use. It was known to ancient Egyptians, and has been used both as a food flavoring and as a traditional medicine. In Ancient Rome, it was "much used for food among the poor". China produces some 80% of the world's supply of garlic. India lies a distant second to China. The garlic plant's bulb is the most commonly used part of the plant. With the exception of the single clove types, garlic bulbs are normally divided into numerous fleshy sections called cloves. Garlic cloves are used for consumption (raw or cooked) or for medicinal purposes like alzheimer's disease, anti-arthritic, antimicrobial, acne- reducers, cold and flu, antifungal, reduces blood pressure Etc (Ademiluyi, 2013).

Ginger is a plant with leafy stems and yellowish green flowers. The ginger spice comes from the roots of the plant. Ginger is native to warmer parts of Asia, such as China, Japan, and India, but now is grown in parts of South American and Africa. It is also now grown in the Middle East to use as medicine and with food. Ginger is commonly used for various types of "stomach problems," including motion sickness, morning sickness, colic, upset stomach, gas, diarrhea, irritable bowel syndrome (IBS), nausea, nausea caused by cancer treatment, nausea caused by HIV/AIDS treatment, nausea and vomiting after surgery, as well as loss of appetite.
Other uses include pain relief from rheumatoid arthritis (RA), osteoarthritis, menstrual pain, and other conditions. However, there is not strong evidence to support the use of ginger for these conditions. (Adetumi, 1986).

Rheumatoid arthritis is a chronic and usually progressive inflammatory disorder of unknown etiology characterized by symmetrical joint involvement and systemic manifestations. It is a chronic, systemic autoimmune disease that involves inflammation in the membrane lining of the joints and often affects internal organs. The antibacterial effect of garlic extract against human dental plaque microbiota has been shown in *in vitro* study. The synergism between ciprofloxacin with garlic extract has been shown, but not between ampicillin and the garlic extracts. The cloves of garlic and rhizomes of ginger, extracted with 95% ethanol, suggested to have anti-bacterial activity against multi-drug clinical pathogens and can be used for prevention of drug resistant

microbial diseases. An antimicrobial is an agent that kills microorganisms or stops their growth. Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. For example, antibiotic are used against bacteria and antifungals are used against fungi. They can also be classified according to their function. (Allison, 2012). Agents that kill microbes are called microbicidal, while those merely inhibit their growth are called biostatic.

Conventional formulations are required to be administered in multiple doses and therefore have several disadvantages like poor patient compliance, fluctuations in drug levels. Recently various strategies have been used to augment the topical delivery of bioactives. Mainly they include iontophoresis, electrophoresis, sonophoresis, chemical permeation enhancers, microneedles, glycerosomes and vesicular drug delivery systems. Among these strategies vesicular drug delivery system appears promising.

Liposomes containing high concentrations of glycerol are known as glycerosomes, which are used in new drug delivery systems. Glycerosomes have greater morphological stability than liposomes.Proteoglycerosomes are defined as glycerosomes that contain membrane proteins. Glycerosomes are modified liposomes containing glycerol in addition to phospholipids. Their special features include the ability to deliver cosmeceutical active ingredients to the skin with high performance, healing, beautification properties. Lately, unilamellar glycerosomes containing quercetin were designed with the size of 80 - 110 nm, which showed to improve skin defensive activity. Glycerosomes and PG-PEVs were obtained using increasing amounts of glycerol or propylene glycol in the water phase (12.5%, 25%, 50% v/v), respectively. (Amagase, 1993).

A liposome is a spherical vesicle having at least one lipid bilayer. The liposome can be used as a vehicle for administration of nutrients and drugs. Liposomes can be prepared by disrupting biological membranes (such as by sonication). Liposomes are most often composed of phospholipids.

Drug carriers used in topical drug delivery such as liposomes, noisome, or micro-emulsions have problem that they remains mostly confined to the skin surface and therefore do not transport drugs efficiently through the skin and this led to intensive research in the field, with the introduction and development of new classes of lipid vesicles. (Ashraf, 2005). Therefore, new, modified formulations have been introduced, such as deformable/elastic liposomes and ethosomes. It is worth noting that glycerosomes containing more than 20% w/w glycerol are more effective than those having lower glycerol (for example from 10 to 15%) in several

respects: A higher glycerol (>20%) concentration improve the rheological properties (e.g. viscosity) and, consequently, give a positive contribute to the stabilization of the glycerosome vesicular system. (Auer W, 1990). Glycerol concentration lower than 20% hamper the 5 assembling of vesicles at room temperature (namely at 25° C.) when phospholipids with high transition temperatures (e.g. 70-80° C.) were used for preparing glycerosomes. Vesicles prepared with a low glycerol concentration (10-15%) display a reduced flexibility and possibly a reduced skin permeation capacity. (Bakhshi, 2012)

TRANSDERMAL DRUG DELIVERYSYSTEM

Conventional systems of medication that require multi-dose therapy are having many problems. The controlled drug delivery is a newer approach is to deliver drug in to systemic circulation at a predetermined rate. Our system should duplicate continuous intravenousinfusion, which not only by passes hepatic 'first pass' elimination but also maintains aconstant, prolonged and therapeutically effective drug level in the body. This is madepossible by using intact skin as a port of drug administration to provide continuous deliveryof drug in to systemic circulation. Following skin permeation, the drugs first reach thesystemic circulation. The drug molecules are then transported to the target site, which could be relatively remote from the site of administration, to produce therapeutic action. (Prausnitz & Langer, 2008)

Transdermal drug delivery offers the following potential advantages.

1. Avoid the risks and inconveniences of intravenous therapy and of varied conditions ofabsorption and metabolism associated with the oral therapy.
2. Continuity of drug administration in TDDS permits the use of a drug with short biological half-life.
3. Transdermal drug delivery improves the bioavailability that reduces the total daily dose.
4. Avoids first-pass hepatic metabolism.
5. Less chances of over or under dosing as the result of prolonged preprogrammed deliveryof drug at the required therapeutic rate.
6. Decrease gastrointestinal side effects.
7. Elimination drug food interactions.

8. Increased patient compliance in following manner
    - Provisions of simplified therapeutic regimen.
    - Painless delivery of drug.
    - Eliminates swallowing.
9. In a multi-drug regimen TDDS avoids drug interaction inGIT.
10. It is easy to terminate the medication simply by removing the drug delivery device fromthe skinsurface.
11. TDDS system can be taken without any aid, which makes it most suitable formulation.
12. Chance of toxicity due to additives is less as compared to other dosageforms.
13. Problem of dose dumping is least in TDDS.
14. Need not to be sterile, obviates and processing problems.

Disadvantages of transdermal drug delivery system:

1. The limitation of transdermal drug delivery is principally associated with skins barrier function, which severely constrains the absolute amount of drug that can be absorbed across reasonable area of skin during the dosing period. Thus, the major disadvantage of the method is that it is limited to potent drug molecule typically those requiring a daily dose on the order of 20 mg or less.
2. Even if the drug is sufficiently potent, it must yet satisfy other criteria to be considered a viable candidate for transdermal drug delivery. For example its physiochemical propertiesmust allow to be absorbed percutaneously.
3. The pharmacokinetic and pharmacodynamic characteristic of the drug must be such that relatively sustained and slow input provided by transdermal delivery makes sense.

Drugs that can be given once a day orally, with reproducible bioavailability and which arewell tolerated by patient do not really need a patch formulation.

Literature Review

Gardner et al., (2001) A meta-analysis including 39 primary trials of the effect of 2 months administration of garlic preparations on total cholesterol, low-density lipoprotein cholesterol,

high-density lipoprotein cholesterol, and triglycerides was performed. The results suggest garlic is effective in reduction of total serum cholesterol by 17±6 mg/dL and low-density lipoprotein cholesterol by 9 ± 6 mg/dL in subjects with elevated total cholesterol levels (>200 mg/dL). An 8% reduction in total serum cholesterol is of clinical relevance and is associated with a 38% reduction in risk of coronary events at 50 years of age. High-density lipoprotein cholesterol levels improved only slightly, and triglycerides were not influenced significantly. Garlic was highly tolerable in all trials and was associated with minimal side effects.

Dini et al., (2011) Garlic has been used for centuries in various societies to combat infectious disease. Historically, it is believed that Louis Pasteur described the antibacterial effect of garlic in 1858 for the first time, although no reference is available. More recently, garlic has been proven to be effective against a plethora of gram-positive, gram-negative, and acid-fast bacteria.

Tsubura et al., (2011) Anti-proliferative activity of ajoene was demonstrated against a panel of human stumor cell lines. Furthermore, allicin inhibits proliferation of human mammary endometrial and colon cancer cells. Growth inhibition is accompanied by an accumulation of the cells in WIG1 and G2lM phase of the cell cycle. Thus allicin is also responsible for the anti-proliferative effect of garlic derivatives. Diallyl sulfide and diallyl disulfide, inhibit arylamine N-acetyltransferase activity and 2-aminofluorene-DNA in human promyelocytic leukemia cells.

Stabler et al., (2012) In one trial, investigation on 47 hypertensive patients showed that garlic significantly decreased the mean systolic blood pressure by 12 mmHg and the mean supine diastolic blood pressure by 9 mmHg versus placebo. The authors stated that garlic was free from side effects and no serious complication was reported.

Zeng et al., (2013) Many in vitro and in vivo studies have suggested possible cancer-preventive effects of garlic preparations and their respective constituents. Garlic has been found to contain a large number of potent bioactive compounds with anticancer properties, largely allylsulfide derivatives. Different garlic derivatives have been reported to modulate an increasing number of molecular mechanisms in carcinogenesis, such as DNA adduct formation, mutagenesis, scavenging of free radicals, cell proliferation and differentiation as well as angiogenesis. The growth rate of cancer cells is reduced by garlic, with cell cycle blockade that occurs in the G2/M phase.

Capasso, (2013) Garlic components have been found to block covalent binding of carcinogens to DNA, enhance degradation of carcinogens, have anti-oxidative and free radical scavenging

properties, and regulate cell proliferation, apoptosis, and immune responses. Ajoene, a garlic stable oil soluble sulfur rich compound and garlic-derived natural compound, have been shown to induce apoptosis in leukemic cells in addition to the other blood cells of leukemic patients. Ajoene induced apoptosis in human leukemic cells via stimulation of peroxide production, activation of caspase-3-like and caspase-8 activity. Garlic synergizes the effect of eicosapentaenoic acid, a breast cancer suppressor, and antagonizes the effect of linoleic acid, a breast cancer enhancer.

Houshmand et al., (2013) The synergism between ciprofloxacin with garlic extract has been shown, but not between ampicillin and the garlic extracts. The cloves of garlic and rhizomes of ginger, extracted with 95% ethanol, suggested to have anti-bacterial activity against multi-drug clinical pathogens and can be used for prevention of drug resistant microbial diseases. Pseudomonas aeruginosa was the most sensitive germ to the mixture.

Chan et al., (2013) Both experimental and clinical studies on different garlic preparations demonstrate these favorable cardiovascular effects. In in vivo animal experiments, intravenous administration of garlic extracts produced slight reductions in both systolic and diastolic pressures. Oral ingestion of garlic extract in hypertensive animals brought the blood pressure back to the normal level. Several clinical studies showed that garlic reduced blood pressure in more than 80% of patients suffering from high blood pressure.

Manca et al., (2013) Glycerosomes contain a high concentration of glycerol in liposome membranes, providing a new drug delivery system, and they have a high stability and a high fluidity. To achieve increased efficiency in GPCR synthesis, liposome conditions have been shown to be optimized, when compared with previous methods, by using.

Liposome combined with highly concentrated glycerol, known as glycerosome, was used as the drug delivery system. The glycerosome membrane has high fluidity and stability

Ried et al., (2013) However, a few studies using garlic powder, having low allicin yields, failed to show any lipid lowering effects. It has been suggested that different people might have different responses to garlic, thus garlic may be more beneficial for some specific groups.

Manca et al., (2016) High fluidity and stability might stabilize GPCR function, as glycerol induced a dose-dependent increase in size stability of the HRH1 proteoglycerosomes. In previous studies hyaluronan was mostly used as a targeting or conjugating agent with curcumin.

Research Gap Identification

The traditional medicine is larger popularity over allopathic medicine because of the following reasons:

1. Rising costs of medical care.
2. As these are from natural origin, so free from side effects.
3. Goes to root cause and removes it, so that the disease does not occur again.
4. Cure from many obstinate disease.
5. Easy availability of drugs from natural sources.
6. Development of chemical process/technologies for natural products involving extraction, isolation and quantification.
7. Development & standardization of herbal drugs and evaluation of biological activity of medicinal plants
8. The phytopharmacological evaluation of herbal drugs and development of new formulations like antimicrobial and anti arthritis cream/ointments

Objectives

The aim of present investigation is to evaluate antimicrobial and anti arthritic potential of *Allium sativum* and *zingiber officinalis* extracts mediated glycerosomes formulation. With the objective to design, optimize and develop glycerosome which might have efficient anti-arthritis property with no or lesser adverse effects than the existing drug of choice.

The following are the objective of study:

1. Collection, authentication and preparation of ethanolic extracts of *Allium sativum* and *zingiber officinalis* collected from Himachal Pradesh.
2. Design, optimization and formulation *of Allium sativum* and *zingiber officinalis* mediated glycerosomes.
3. Comparative evaluation of antimicrobial and anti arthritic activity of *Allium sativum* and *zingiber officinalis* extract mediated glycerosomes.
4. *Allium sativum* and *zingiber officinalis* glycerosomes compared with marketed glycerosomes formulation of celecoxib (CLX) and cupferron (CUP).

Proposed Methodology

The present study will be carried out to study antimicrobial and anti arthritic potential of *Allium sativum* and *zingiber officinalis*e xtracts mediated glycerosomes formulation, collected Himachal Pradesh. (Banejee, 2002). (30°22'40s″- 33°12′40″ N to 75°45′55″- 79°04′20″ E).

Material:

Chemicals and solvents used: All chemicals used will of analytical grade and obtained commercially. (Bordia, 1989). The synthetic media will be used for culturing of bacteria obtained from Himedia Labs, Mumbai. The solvents such as ethanol, methanol, choloroform, ethyl acetate, butane lwill be purchased from SD Fine chem. pvt. Ltd, Haryana, India.

Microbial strains: The four microbial strains (two gram +vei. E *Staphylococcus aureus* and *Bacillus subtilis* and two gram –ve *Escherichia coli and pseudomonas aeruginosa*) will be used to study antimicrobial activity of*Allium sativum* and *zingiber officinalis*extracts mediated glycerosomes. (Cai, 1991). All the strains were obtained from Department of Microbiology, Career Point University, Hamirpur, Himachal Pradesh.)

Growth media: The bacterial strains will be cultured in nutrient agar at 37 ̊C [(A., 2013)] growth media purchased from Merck Pvt. Ltd, India. Amoclave (10µg) will be used as positive control for antimicrobial activity. (Cavallito C.J, 1944).

Table No.1:- List of equipments used in the study

Sr. No.	Instrument / Equipment
1	Magnetic stirrer
2	Melting point apparatus
3	pH meter
4	Sonicator
5	Soxhlet Apparatus
6	Homogenizer
7	Digital Balance
8	Seive Shaker
9	Heating Mentle
10	Transmission Electron Microscope

11	UV-Visible spectrophotometer
12	Water bath shaker
13	Viscometer
14	Laminar air flow
15	Bacterial incubator
16	Autoclave
17	Hot air oven
18	Antibiotic zone reader

Table No.2:- List of Software's used in the study

Sr. No	**Software used in the study**
1	GraphPad Prism

Methodology:

Collection and Preparation of plant extract: The clove of garlic and zinger will be collected local market of Hamirpur district of Himachal Pradesh. Extraction is the separation of medicinally active portions of plant using selective solvents through standard procedures. (Chanderkar, 1973). Ethanolic extracts of all selected plants will be prepared. For the preparation of ethanolic extract of each plant samples, 500gram powder of each sample was weighed and is then subjected to soxhlet extractor. (Colin-Gonzalez, 2012). Defating with touline is done for 48hrs. After defating moist powdered sample is taken out from extractor and dried in hot air oven for touline to get evaporated. (Davis, 2003). When the touline get totally evaporated, dried powdered sample is again subjected to extractor. Ethanol was added as a solvent to the sample.Then the extractor was allowed to run for next 48hrs at ethanol's boiling point i.e. 78.3˚c. (De Witt, 1979). These extracts were stored at 4˚c for further use.

Qualitative Phytochemical Screening: The different crude extract will be examined for the presence of various phytochemical constituents such as phenols, flavonoids, tannin, saponin, alkaloids, glycosides and carbohydrate by using standard protocols. (Delaha, 1985).

Table No.3:- List of different tests used for Phytochemical Screening.

Sr. No.	Phytoconstituents	Test
1.	Alkaloids	Dragendroff's test Hager test Mayer test Wagner test
2.	Tannins	Alkaline reagent test Ferric chloride test Gelatin test Lead acetate test
3	Phenol	Ferric chloride test Gelatin test Lead acetate test
4	Phytosteroids	Liebermann-Burchard's test
5	Phytosterol	Salkowski reaction test
6.	Saponin	Foam test
7.	Carbohydrates	Benedict test Barfoed test Fehling test
8	Cardiac Glycosides	Kedde's test Keller-Killiani test Legal test Guignard's test
9.	Proteins	Biuret test Millon test Ninhydrin test
10	Flavonoids	Alkaline reagent test Lead acetate test Shinoda test Vanillin-HCl test

11	Gum and Mucilage	Ruthenium red test Molisch's test Test with 95% alcohol
12	Fixed oil and Fats	Fat test
13	Anthraquinone glycosides	Borntrager's test Modified Borntrager's test

Preparation of Glycerosomes:-

Glycerosomes and liposomes were prepared according to the lipidic film hydration technique. Briefly, weighted lipidic components (phospholipids, cholesterol, hydrophobic active principles or ionic lipids such as DCP or stearylamine) were dissolved in 5 ml of organic solvent (chloroform or dichlo-romethane) using a 50 ml round bottom flask containing 4-5 ml of 1 mm diameter glass beads. Solvent was removed using a rotary evaporator under vacuum at room temperature for 2 hours to get on the wall of the flask a lipidic film free of any trace of solvent. 5 ml of glycerol aqueous solution (glycerol concentration between 20% and 35%) also containing, if required, water soluble active principles were then added and the mixture was maintained under mechanical agitation at 10.000 rpm for 1 hour at 25° C. using a crescent-shaped plastic stirring shaft in close contact with the internal round bottom flask surface. The emulsion, consisting of multilamel- lar vesicles, was maintained at rest for 1 hour and then homogenized at high-pressure for 10 minutes at 25° C. (18. Dini, 2011). using a Avestin Emulsiflex C5 homogenizer set at 60,000-65,000 kPa. Homogenated vesicles were purified by gel filtration on a Sephadex® G25 column, a bead-formed gel prepared by cross-linking dextran with epichlorohydrin, to separate glycerosomes, which were eluted in void volume, from non incorporated active principle. Column elution was carried out with water and the collected fractions were checked by UV turbi- dimetric analysis at 600 nm to identify the vesicle rich fractions. (Fenwick, 1985).

Conventional liposomes were prepared as described but performing lipidic film hydration with aqueous buffer (for example with saline phosphate buffer); besides, both hydration and next homogenization phases were carried out at a temperature corresponding to or higher than the

transition temperature (Tc) of the phospholipidic component (for example DPPC with Tc=70° C.; DMPC with Tc=25° C.; etc). (Fromtling, 1978).

Glycerosomes Characterization:-

The appearance of the STO-glycerosomes was examined using a transmission electron microscope. Samples were prepared for negative staining as follows: glycerosomes were diluted with a 10% (v/v) glycerol–water solution and gently dropped on a film-coated copper grid (Zhong Jing Ke Yi Technology Inc., Beijing, People's Republic of China). After 20 min of drying, a drop of phosphotungstic acid (2%, v/v) was added to the film. After air-drying for 3 h at room temperature, the film was observed under a transmission electron microscope. (Gardner, 2001).

Antimicrobial activity: - The antimicrobial activity for different extracts will be determined by the disc diffusion method (Bauer *et al*., 1966). Both gram positive and gram-negative bacterial strains along with pathogenic fungal strains will be used for the test. Solutions of known concentration (mg/ml) of the test samples will be made by dissolving measured amount of the samples in calculated volume of solvents. (Gebhardt, 1996). Dried and sterilized filter paper discs will then impregnated with known amounts of the test substances using micropipette. Discs containing the test material will be placed on nutrient agar medium uniformly seeded with the test microorganisms.These plates will then incubate at 37ºC for 24 h to allow maximum growth of the organisms. The test materials having antimicrobial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition will be visualized surrounding the medium. The antimicrobial activity of the test agents will be determined by measuring the diameter of zone of inhibition expressed in millimeter. (Ghannoum, 1988).

In *Vitro* anti-arthritic activity:-

1. **Inhibition of protein denaturation method:** The reaction mixture (0.5 ml) consisted of 0.45 ml bovine serum albumin (5% aqueous solution) and 0.05 ml of plant extract (ethanolic) at different concentration. Then the samples were incubated at 37°C for 30 min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube and the turbidity was measured spectrophotometrically at 660 nm for control test 0.05 ml distilled water was used instead of extracts while product control test lacked bovine serum albumin. (Manca ML, 2013). The percentage inhibition of protein denaturation was calculated as follows:

$$Percentage\ inhibition = \frac{Abs_{control} - Abs_{treated}}{Abs_{treated}} \times 100$$

2. Inhibition of albumin denaturation: The 5 ml of reaction mixture was comprised of 0.2 ml of eggs albumin (from hens egg), 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentration of ethanolic extracts and similar volume of double distilled water was served as control. Then the mixture was incubated at 37°C in BOD incubator for about 15 mins and then heated at 70°C for 5 mins. After cooling, their absorbance was measured at 660 nm by using pure blank. Diclofenac sodium (Standard drug) was used as reference drug and treated as such for determination of absorbance. (Manca, 2016).

Impact of the research work on Society/Academics/Industry:-

1. Conventional formulations are required to be administered in multiple doses and therefore have several disadvantages like poor patient compliance, fluctuations in drug levels.
2. Glycerosome are include the ability to deliver cosmeceutical active ingredients to the skin with high performance, healing, beautification properties
3. Production of standard quality drugs in Ayurvedic pharmacythe need to produce the standard quality drugs in Ayurveda.
4. To design, optimize and develop semi solid dosage form which might have efficient antimicrobial and anti-arthritic property with no or lesser adverse effects than the existing drug of choice.

Thesis Outline

Chapter 1: Introduction. It covers the aim and objectives of research topic i.e. Invitro evaluation of Allium Sativum and Zingiber Officinalis extracts mediated Glycerosomes for antimicrobial and antiarthritic potential.

Chapter 2: Literature Review. It cover review of work done on Transdermal drug delivery system.

Chapter 3: Materials and Methods. It covers the list of material, equipments and methods used in research work.

Chapter 4: Result and discussion. It covers the preformulation, evaluation results of Glycerosomes.

Chapter 5: Summary and conclusion. It covers the concise statement and key point of Transdermal drug delivery system.

Chapter 6: References. It coves every source. The reference list entries contain four main component: author, date, title and source.

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CERTIFICATE
(SYNOPSIS APPROVAL CERTIFICATE)

This is to certify that the Synopsis/Revised Synopsis for Ph.D. Thesis titled, **"InVitro evaluation of *Allium Sativum* and *Zingiber Officinalis* extracts mediated glycerosomes for antimicrobial and anti-arthritic potential",** submitted by **Ms. Kiran Thakur** to the Career Point University, Hamirpur (H.P.), India in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in the Division/**Department of Pharmaceutical Sciences, School of Pharmaceutical and Health Sciences** has been approved by the Research Advisory Committee (RAC) after an Oral Examination of the student on dated 15/01/22.

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